IDENTIFICATION OF AN ALTERNATE HOST FOR *BABESIA BOVIS* AND *BABESIA BIGEMINA*

A Senior Scholars Thesis

by

MAEGAN M. RAMOS

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2011

Major: Biomedical Sciences

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Approved by:

Research Advisor: Patricia J. Holman
Director for Honors and Undergraduate Research: Sumana Datta

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ABSTRACT

Identification of an Alternate Host for *Babesia bovis* and *Babesia bigemina*. (April 2011)

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In the late 1800s, a disease known as "Texas Cattle Fever" became an economic and epidemiologic problem for cattle ranchers in Texas and parts of the southern United States. A tick-borne hemoparasite known as *Babesia* which infects red blood cells of its hosts is the cause of this disease, which is sometimes also referred to as bovine babesiosis. Tick vectors of *Rhipicephalus* (*Boophilus*) spp. carry the *Babesia* species and a 37 year eradication effort known as the Cattle Fever Tick Eradication Program was conducted to rid the country of these vectors. A Cattle Fever Tick quarantine buffer zone has been maintained since along the Texas-Mexico border, however, since this neighboring country failed to eradicate the tick, there is a threat of possible re-introduction and re-establishment of Cattle Fever Ticks in the U.S. The occurrence of fever tick outbreaks has become more frequent within recent years and the location of these outbreaks is moving further northward from the Texas-Mexico border. It is suspected that white-tailed deer and other wildlife ungulates are possible hosts of the blood-borne parasites, *Babesia bovis* and *Babesia bigemina*, which cause bovine

babesiosis. Previous studies conducted in several Mexican states bordering Texas, as well as in counties in South Texas, suggest that both *B. bovis* and *B. bigemina* may be carried by white-tailed deer. The purpose of this research was to determine whether white-tailed deer served as reservoirs for *B. bovis* and *B. bigemina*. Approximately 232 white-tailed deer blood samples were provided for this study. Samples were screened by specific PCR for these parasites and those positive for the presence of *B. bovis* and *B. bigemina* parasites were cloned, sequenced and analyzed. The resulting sequences from white-tailed deer parasites showed 99% identity to known bovine *Babesia* spp. indicating that these parasites are very closely related to those found in cattle. Further work is needed to determine if the *Babesia* spp. found in white-tailed deer are infective to cattle.

DEDICATION

I would like to dedicate this senior scholars thesis to my mother, Doris Ramos, and my father, Raul Ramos Jr., without whom I would not be here today. I would also like to dedicate this thesis to my loving siblings, Marina and Raul Ramos III, and boyfriend, Michael Lazo, who have shown unending support throughout my four years at Texas A&M University.

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This study was supported by Texas AgriLife Research Project H8987 (Patricia J. Holman).

NOMENCLATURE

APHIS	Animal and Plant Health Inspection Service
DNA	Deoxyribonucleic Acid
ITS	Intervening Transcribed Spacer
PAUP	Phylogenetic Analysis Using Parsimony
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
USDA	United States Department of Agriculture
WTD	White-tailed Deer

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CHAPTER I

INTRODUCTION

During the late 1800s, infection of cattle in Texas and parts of the southern United States with a disease known as "Texas Cattle Fever" became an economic and epidemiologic problem for cattle ranchers. Clinical signs of Texas Cattle Fever include anemia, lethargy, emaciation, reduced lactation, fever, and eventually death in susceptible livestock. The disease, which is sometimes referred to as bovine babesiosis, is a serious tick borne disease resulting from a hemoparasite known as *Babesia* which infects red blood cells of its hosts. The *Babesia* species are carried by tick vectors of the *Rhipicephalus (Boophilus)* spp. for which a mass eradication effort known as the Cattle Fever Tick Eradication Program was made beginning in 1906 and ending in 1943 with successful eradication of the vector tick species in the United States (Graham and Hourrigan, 1977). A Cattle Fever Tick quarantine buffer zone is maintained along the Texas-Mexico border, however, since this neighboring country failed to eradicate the tick.

Since the vector ticks remain enzootic in Mexico, there is a threat of possible re-introduction and re-establishment of Cattle Fever Ticks in the U.S. During the 1970s, vector tick outbreaks occurred in Texas in areas both within and outside of the buffer

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This thesis follows the style of Veterinary Parasitology.

zone, causing concern that a similar pattern of re-establishment could occur (Perez de Leon, et al., 2010). This resulted in six years of eradication efforts and significant costs for cattle ranchers. Within recent years a similar pattern of fever tick findings has occurred with increasing frequency and increasing movement northward from the Texas-Mexico border (Perez de Leon, et al., 2010). White-tailed deer and other wildlife ungulates, which are known to carry the vector tick, are under suspicion as possible silent carriers of the blood-borne parasites, *Babesia bovis* and *Babesia bigemina,* which cause bovine babesiosis (Fig. 1).

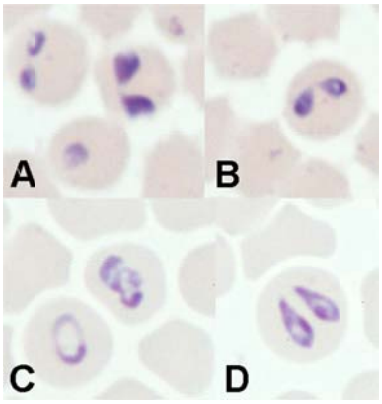

Fig 1. *Babesia bovis* (A, B) and *Babesia bigemina* (C, D) parasitized bovine red blood cells. *Babesia bovis* and *B. bigemina* are known causative agents of bovine babesiosis. Giemsa stain.

Due to the increasing number of wildlife ranches maintained for hunting, high populations of white-tailed deer and other ungulate hosts are being sustained, which will likely lead to an increase of *Rhipicephalus (Boophilus)* spp. tick populations in non-

buffer zone areas of southern Texas. During 2009, it was reported that approximately 30% of cattle fever tick occurrences in the buffer zone were attributable to white-tailed deer (Perez de Leon, et al., 2010).

As the tick outbreaks become more frequent, the risk of re-establishment of bovine babesiosis in the U.S. also increases. In 1943, when Cattle Fever Ticks were eradicated from the U.S., these tropical ticks were only able to survive in the southern states. Now, due to the climate changes causing higher temperatures in a greater portion of the U.S., fever ticks are able to proliferate in areas that were previously too cold for their survival (George, 2008). This means that if the tick becomes re-established in the U.S., it will have an even greater impact on the cattle industry than in the past.

Available pesticides for Cattle Fever Ticks are becoming obsolete due to pesticide resistance in the ticks resulting from misapplication of acaricides for cattle tick control in Mexico (Miller et al., 2007). There are no vaccines against bovine babesiosis, there are no anti-babesial drugs approved for use in U.S. cattle, and the limited alternatives for treatment options are very expensive and often unsuccessful. Re-establishment of Cattle Fever Ticks and the potential for the re-emergence of bovine babesiosis present a serious threat to the Texas cattle industry. It is estimated that the economic impact on the livestock industry could reach $3 billion if *Babesia*-infected *Boophilus* become re-established in Texas (Bram et al., 2002).

White-tailed deer are known hosts for the tick that vector the protozoan parasites that cause bovine babesiosis, leading to investigations of their role as potential reservoirs of the *Babesia* spp. as well. Reports from several Mexican states bordering Texas, as well as from counties in South Texas, show molecular and serologic evidence of both *B. bovis* and *B. bigemina* in white-tailed deer (Cantu et al., 2007, 2009; Ramos et al., 2010). These studies have fueled fears that white-tailed deer in the U.S. may serve as reservoirs of infection for cattle.

The true identity of the deer parasites remains unknown. To date, the evidence for their existence is based solely on serology and *Babesia* DNA detection. The parasitemias in the deer are below detectable levels by microscopy, so the morphology of the deer parasites is unknown. Recent studies using immune fluorescent antibody assays and "specific" polymerase chain reaction (PCR) assays for *B. bovis* and *B. bigemina* in the laboratory of the PI have shown that white-tailed deer do, indeed, carry *Babesia* species that are similar to the bovine parasites (Ramos et al., 2010). However, these results are inconclusive as to whether the deer parasites are actually the same as the bovine parasites and are infective to cattle. The IFA test is not infallible for identification purposes as cross-reactions between related *Babesia* species are known to occur (Orinda et al., 1992). Follow-up sequence analysis of the positive products in *B. bovis* and *B. bigemina* "specific" PCR based on the 18S ribosomal RNA gene reveals that the deer parasites are slightly different in 18S rRNA gene sequence from bovine isolates (Ramos

et al., 2010). Whether this differentiates the deer parasites as species distinct from the bovine parasites must be investigated further.

I hypothesize that white-tailed deer do in fact serve as reservoirs of infection for bovine babesiosis. The aim of this research is to determine whether white-tailed deer serve as reservoirs for *B. bovis* and *B. bigemina*. Samples positive for the presence of the *B. bovis and B.bigemina* -like parasite will be determined by an 18S ribosomal RNA gene-based PCR assay of all 232 WTD blood samples provided for the study. Comparative gene analysis of 18S rDNA will be used to molecularly characterize the parasites. To further characterize the deer isolates, the ribosomal DNA region spanning the intervening transcribed spacer 1, 5.8S rRNA gene, and ITS 2 (referred to as ITS throughout) will be sequence analyzed in deer *B. bovis*-like parasites and compared to known bovine *B. bovis* isolates.

CHAPTER II

METHODS

Samples

Blood samples in ethylenediamine tetraacetic acid-K3 (EDTA) collected from white-tailed deer (WTD) during capture and release of a separate ongoing project (USDA, Dr. A. Pérez de León; APHIS project, Dr. G. Schuster; Texas AgriLife-Uvalde, Dr. S. Cooper) were provided to our laboratory for the purpose of this study. One hundred sixty-nine samples were collected from three south Texas areas in Zapata County. Of these, 72 samples were collected from area HF, 33 samples were collected from area SL, and 34 samples were collected from area NL. Thirty samples provided by the USDA, also from Zapata County, were identified as C, I, or R. In addition, 63 samples from Webb County were provided by the Texas AgriLife project and were identified as either W or L (Fig. 2).

To extract DNA, 200 µl blood samples were centrifuged at 500 x *g* for 15 minutes to allow cells to pellet. The cell pellet was washed once by centrifugation in 10 volumes of Dulbecco's phosphate buffered saline PBS. DNA extraction of the cell pellets was performed according to manufacturer's instructions (FlexiGene DNA Extraction,

Fig 2. Blood samples were collected from two south Texas counties, Webb County, shown here in blue and Zapata County, shown here in green. One hundred sixty-nine samples were collected from WTD in Zapata County and 63 samples were collected from WTD in Webb county.

Qiagen, Valencia, CA). The cell pellet was resuspended in lysis buffer and centrifuged at 10,000 x *g*, after which the supernatant was removed. The pellet was resuspended in a buffer containing protease, vortexed, and heated at 65°C for 5 minutes to allow protein digestion. After isopropanol was added to the sample, the sample was inverted and a

DNA precipitate formed. The sample was then centrifuged at 10,000 x *g* for 3 min and the supernatant was discarded. A solution of 70% ethanol was added to wash the remaining pellet and the sample was centrifuged once more for 3 min at 10,000 x *g*. The supernatant was discarded and a final buffer was added to the DNA. The DNA was allowed to resuspend and DNA concentrations were measured (Nanodrop ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE).

PCR amplification of *Babesia* 18s rRNA gene segment

A primary PCR was performed in order to amplify the full-length parasite 18S rRNA gene using primer A (forward primer, 5'- AACCTGGTTGATCCTGCCAG-3') and primer B (reverse primer, 5'-GATCCTCTGCAGGTTCACCTAC-3') (Sogin, 1990). This amplifies all hemoparasite 18S rRNA genes. It was followed by a nested PCR in order to test for the presence of a particular hemoparasite species.

Amplifications were performed using a high fidelity *Taq* polymerase (High Fidelity Platinum *Taq*, Invitrogen, Carlsbad, CA) with a final PCR volume of 12.5 µl, following manufacturer's instructions. Each reaction contained 50-100 ng of genomic DNA in a 12.5 µl volume composed of 1 X High Fidelity PCR Buffer, 0.2 mM dNTP, 2 mM $MgSO_4$, 0.2 µM of each primer A and B and 0.25 U *Taq* polymerase. The primary PCR amplification profile was as follows: initial denaturation at 94 °C for 30 s, 45 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 15 s, extension at 68 °C for 2 min, final extension at 68 °C for 7 min and a hold at 4 °C (Labnet MultiGene Thermal Cycler,

Woodbridge, NJ). PCR products were stained with ethidium bromide and viewed under ultraviolet transillumination after electrophoresis through 1% agarose gels alongside a 100 base pair marker (Invitrogen, Carlsbad, CA).

Primers to specifically amplify *B. bovis* (Table 1) 18s rRNA genes were previously described (Ramos et al., 2010). *Babesia bigemina* primers (Table 1) were designed based on *B. bigemina* 18S rRNA gene sequences found in the GenBank database (NCBI). The nested PCR reactions were performed using appropriate dilutions of the primary PCR amplification products (1:5, 1:10 or 1:20 depending on band intensity of the primary PCR amplicons viewed in agarose gels) as template DNA and appropriate positive and negative control plasmid DNA. The amplification profile was as above, but reduced to 30 cycles with 1 min 30 s extension and annealing temperatures (Table 1).

Table 1

Oligonucleotide primer sets for nested PCR to amplify 18S rDNA from *Babesia bovis* or *Babesia bigemina*

Target 18S rDNA	Primer	Nucleotide Sequence	Annealing Temperature
Babesia bovis	Bbov600F	5'-gcttggtcctttcctcgc-3'	56 ºC
	Bbov1500R	5'-gatcgcgcaagcaggttc-3'	56 ºC
Babesia bigemina	Bbig200F	5'-gcgtttattagttcgttaacc-3'	56 ºC
	Bbig1400R	5'-acaggacaaactcgatggatgc-3'	56 ºC

Four samples found positive through *B. bovis* 18S rRNA PCR were further characterized by PCR specific for the rRNA ITS1-5.8S gene-ITS2 region (Table 2). The primary PCR amplification profile was as follows: initial denaturation at 94 °C for 30 s, 45 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 15 s, extension at 68 °C for 2 min, final extension at 68 °C for 7 min and a hold at 4 °C (Labnet MultiGene Thermal Cycler, Woodbridge, NJ). PCR products were stained with ethidium bromide and viewed under ultraviolet transillumination after electrophoresis through 1% agarose gels alongside a 100 base pair marker (Invitrogen). A nested PCR was then performed using primers specific for the *B. bovis* ITS region as described above. The PCR amplification was the same as for the primary ITS PCR, but reduced to 30 cycles with 1 min 30 s extension and annealing temperatures as shown in Table 2.

Table 2

Oligonucleotide primer sets for primary and nested PCR to amplify rRNA ITS1-5.8S gene-ITS2 region from *Babesia bovis*

Target	Primer	Nucleotide Sequence	Annealing Temperature
Primary ITS	528EXTF	5'-cggtaattccagctccaatagc-3'	55 ºC
Primary ITS	BboLsuR	5'-cttgtctgccgcttagttatagc-3'	55 ºC
Nested *B. bovis* ITS	Bbo1600F	5'-tgcgcgatccgtcg-3'	55 ºC
Nested *B. bovis* ITS	BboLsuRN	5'-ggatagcctcgtacatctcagg-3'	55 ºC

Cloning and sequencing of *Babesia* PCR products

Cloning followed manufacturer's instructions (TOPO TA Cloning kit, Invitrogen) to incorporate amplicons into the TOPO 2.1 plasmid, and to transform TOP 10 competent *Escherichia coli*. Transformation was confirmed through colony PCR and positive colonies were selected for plasmid DNA extraction. The selected colonies were grown overnight in Luria Bertoni broth containing 50 µg/ml kanamycin in preparation for plasmid extraction the following morning (QIAprep Spin Miniprep, Qiagen, MD). The concentration of plasmid DNA was measured (NanoDrop ND-1000 Spectrophotometer) and sequenced (Davis Sequencing, Inc. Davis, CA) using M13 forward and M13 reverse primers. The 18S rRNA sequences acquired were aligned; analyzed and contiguous sequences determined using Sequencher 4.2 software (Gene Codes Corporation, Inc., Ann Arbor, MI). The contiguous sequences were compared to gene sequences in the GenBank database using the NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Alignment of sequences obtained throughout this project was conducted using Clustal W2 (Larkin et al., 2007). Sequences obtained during this study were deposited in the NCBI GenBank Database.

Aligned sequences were used to create a phylogenetic tree using the Neighbor-Joining algorithm in PAUP* version 4.0b10 (Swofford, 2002) with molecular distances estimated by the Kimura two parameter model (Kimura, 1980).

CHAPTER III

RESULTS

There were 169 samples from Zapata County and 63 samples from Webb County that were tested by *B. bovis* and *B. bigemina* specific PCR. Four of the 30 USDA samples (13.3%) from Zapata County, Texas were found to be positive by *B. bovis* PCR (Table 3). Five of the 63 Webb County "W" samples (20.8%), and four of the 138 Zapata County samples (2.90%) from the APHIS project were found to be positive by PCR for *B. bovis*. Three of the 24 Webb county "W" samples (12.5%) and one of the 30 USDA samples (3.33%) from Zapata County were also found to be PCR positive for *B. bigemina*. Four additional Webb County samples positive by *B. bovis* PCR (data not shown) were further characterized by amplifying the parasite rRNA ITS 1- 5.8S rRNA gene-ITS 2 genomic region (ITS). All *B. bovis* and *B. bigemina* 18S rDNA, and *B. bovis* ITS PCR positive samples were subsequently cloned and sequenced.

The resulting *B. bovis* 18S rDNA sequences showed 99% identity to *B. bovis* 18S rDNA sequences of cattle origin in the GenBank database. A phylogram was created (Swofford, 2002), which included sequences from known bovine *B. bovis* 18S rDNA isolates from the GenBank database (EU073963; BboMer1, GU906883.1; BboMer2, GU906884.1), illustrating the relationships among the sequences (Fig. 3). The neighbor-joining phylogram and bootstrap trees showed correlation between the origin

of the samples sequenced and grouping of the samples into the various branches. There were some

Table 3

PCR results for white-tailed deer blood samples. A "+" symbol indicates a sample was positive and a "0" indicates a sample was negative.

Date	Project Source	Animal I.D.	County	*B. bovis*	*B. bigemina*
July 2010	APHIS	HF 1	Zapata	0	0
July 2010	APHIS	HF 2	Zapata	0	0
July 2010	APHIS	HF 3	Zapata	0	0
July 2010	APHIS	HF 4	Zapata	0	0
July 2010	APHIS	HF 5	Zapata	0	0
July 2010	APHIS	HF 6	Zapata	0	0
July 2010	APHIS	HF 7	Zapata	0	0
July 2010	APHIS	HF 8	Zapata	0	0
July 2010	APHIS	HF 9	Zapata	0	0
July 2010	APHIS	HF 10	Zapata	0	0
July 2010	APHIS	HF 11	Zapata	0	0
July 2010	APHIS	HF 12	Zapata	0	0
July 2010	APHIS	HF 13	Zapata	0	0
July 2010	APHIS	HF 14	Zapata	0	0
July 2010	APHIS	HF 15	Zapata	0	0
July 2010	APHIS	HF 16	Zapata	0	0
July 2010	APHIS	HF 17	Zapata	0	0
July 2010	APHIS	HF 18	Zapata	0	0
July 2010	APHIS	HF 19	Zapata	0	0
July 2010	APHIS	HF 20	Zapata	0	0
July 2010	APHIS	HF 21	Zapata	+	0
July 2010	APHIS	HF 22	Zapata	0	0
July 2010	APHIS	HF 23	Zapata	0	0
July 2010	APHIS	HF 24	Zapata	0	0
July 2010	APHIS	HF 25	Zapata	0	0
July 2010	APHIS	HF 26	Zapata	0	0
July 2010	APHIS	HF 27	Zapata	0	0
July 2010	APHIS	HF 28	Zapata	0	0
July 2010	APHIS	HF 29	Zapata	0	0
July 2010	APHIS	HF 30	Zapata	0	0
Oct. 2010	APHIS	HF101	Zapata	0	0
Oct. 2010	APHIS	HF103	Zapata	0	0
Oct. 2010	APHIS	HF104	Zapata	0	0
Oct. 2010	APHIS	HF105	Zapata	0	0
Oct. 2010	APHIS	HF106	Zapata	0	0

Table 3 continued

Oct. 2010	APHIS	HF107	Zapata	0	0
Oct. 2010	APHIS	HF108	Zapata	0	0
Oct. 2010	APHIS	HF109	Zapata	0	0
Oct. 2010	APHIS	HF110	Zapata	0	0
Oct. 2010	APHIS	HF111	Zapata	0	0
Oct. 2010	APHIS	HF113	Zapata	0	0
Oct. 2010	APHIS	HF114	Zapata	0	0
Oct. 2010	APHIS	HF115	Zapata	0	0
Oct. 2010	APHIS	HF116	Zapata	0	0
Oct. 2010	APHIS	HF117	Zapata	0	0
Oct. 2010	APHIS	HF118	Zapata	0	0
Oct. 2010	APHIS	HF119	Zapata	0	0
Oct. 2010	APHIS	HF121	Zapata	0	0
Oct. 2010	APHIS	HF122	Zapata	0	0
Oct. 2010	APHIS	HF123	Zapata	0	0
Oct. 2010	APHIS	HF126	Zapata	0	0
Oct. 2010	APHIS	HF127	Zapata	0	0
Oct. 2010	APHIS	HF128	Zapata	0	0
Oct. 2010	APHIS	HF129	Zapata	0	0
Oct. 2010	APHIS	HF130	Zapata	0	0
Oct. 2010	APHIS	HF131	Zapata	0	0
Oct. 2010	APHIS	HF132	Zapata	0	0
Oct. 2010	APHIS	HF133	Zapata	0	0
Oct. 2010	APHIS	HF134	Zapata	0	0
Oct. 2010	APHIS	HF135	Zapata	0	0
Oct. 2010	APHIS	HF136	Zapata	0	0
Oct. 2010	APHIS	HF137	Zapata	0	0
Oct. 2010	APHIS	HF138	Zapata	0	0
Oct. 2010	APHIS	HF139	Zapata	0	0
Oct. 2010	APHIS	HF140	Zapata	0	0
Oct. 2010	APHIS	HF141	Zapata	0	0
Oct. 2010	APHIS	HF142	Zapata	0	0
Oct. 2010	APHIS	HF143	Zapata	0	0
Oct. 2010	APHIS	HF144	Zapata	0	0
Oct. 2010	APHIS	HF145	Zapata	0	0
Oct. 2010	APHIS	HF146	Zapata	0	0
Oct. 2010	APHIS	HF147	Zapata	0	0
July 2010	APHIS	SL2	Zapata	+	0
July 2010	APHIS	SL3	Zapata	0	0
July 2010	APHIS	SL4	Zapata	0	0
July 2010	APHIS	SL5	Zapata	0	0
July 2010	APHIS	SL6	Zapata	0	0

Table 3 continued

July 2010	APHIS	SL7	Zapata	0	0
July 2010	APHIS	SL8	Zapata	0	0
July 2010	APHIS	SL9	Zapata	0	0
July 2010	APHIS	SL10	Zapata	+	0
July 2010	APHIS	SL11	Zapata	+	0
July 2010	APHIS	SL12	Zapata	0	0
July 2010	APHIS	SL13	Zapata	0	0
July 2010	APHIS	SL14	Zapata	0	0
July 2010	APHIS	SL15	Zapata	0	0
Oct. 2010	APHIS	SL61	Zapata	0	0
Oct. 2010	APHIS	SL62	Zapata	0	0
Oct. 2010	APHIS	SL63	Zapata	0	0
Oct. 2010	APHIS	SL64	Zapata	0	0
Oct. 2010	APHIS	SL65	Zapata	0	0
Oct. 2010	APHIS	SL66	Zapata	0	0
Oct. 2010	APHIS	SL67	Zapata	0	0
Oct. 2010	APHIS	SL68	Zapata	0	0
Oct. 2010	APHIS	SL69	Zapata	0	0
Oct. 2010	APHIS	SL71	Zapata	0	0
Oct. 2010	APHIS	SL72	Zapata	0	0
Oct. 2010	APHIS	SL73	Zapata	0	0
Oct. 2010	APHIS	SL74	Zapata	0	0
Oct. 2010	APHIS	SL75	Zapata	0	0
Oct. 2010	APHIS	SL76/96	Zapata	0	0
Oct. 2010	APHIS	SL77	Zapata	0	0
Oct. 2010	APHIS	SL78	Zapata	0	0
Oct. 2010	APHIS	SL79	Zapata	0	0
Oct. 2010	APHIS	SL80	Zapata	0	0
July 2010	APHIS	NL1	Zapata	0	0
July 2010	APHIS	NL2	Zapata	0	0
July 2010	APHIS	NL3	Zapata	0	0
July 2010	APHIS	NL4	Zapata	0	0
July 2010	APHIS	NL5	Zapata	0	0
July 2010	APHIS	NL6	Zapata	0	0
July 2010	APHIS	NL7	Zapata	0	0
July 2010	APHIS	NL8	Zapata	0	0
July 2010	APHIS	NL9	Zapata	0	0
July 2010	APHIS	NL10	Zapata	0	0
July 2010	APHIS	NL11	Zapata	0	0
July 2010	APHIS	NL13	Zapata	0	0
July 2010	APHIS	NL14	Zapata	0	0
July 2010	APHIS	NL15	Zapata	0	0
Oct. 2010	APHIS	NL81	Zapata	0	0

Table 3 continued

Oct. 2010	APHIS	NL82	Zapata	0	0
Oct. 2010	APHIS	NL83	Zapata	0	0
Oct. 2010	APHIS	NL84	Zapata	0	0
Oct. 2010	APHIS	NL85	Zapata	0	0
Oct. 2010	APHIS	NL86	Zapata	0	0
Oct. 2010	APHIS	NL87	Zapata	0	0
Oct. 2010	APHIS	NL88	Zapata	0	0
Oct. 2010	APHIS	NL89-1	Zapata	0	0
Oct. 2010	APHIS	NL89-2	Zapata	0	0
Oct. 2010	APHIS	NL90	Zapata	0	0
Oct. 2010	APHIS	NL91	Zapata	0	0
Oct. 2010	APHIS	NL92	Zapata	0	0
Oct. 2010	APHIS	NL94	Zapata	0	0
Oct. 2010	APHIS	NL95	Zapata	0	0
Oct. 2010	APHIS	NL96	Zapata	0	0
Oct. 2010	APHIS	NL97	Zapata	0	0
Oct. 2010	APHIS	NL98	Zapata	0	0
Oct. 2010	APHIS	NL99	Zapata	0	0
Aug. 2010	USDA	1C	Zapata	0	0
Aug. 2010	USDA	2C	Zapata	+	0
Aug. 2010	USDA	3C	Zapata	0	0
Aug. 2010	USDA	4C	Zapata	+	0
Aug. 2010	USDA	5C	Zapata	+	0
Aug. 2010	USDA	6C	Zapata	0	0
Aug. 2010	USDA	7C	Zapata	0	0
Aug. 2010	USDA	8C	Zapata	0	0
Aug. 2010	USDA	9C	Zapata	0	0
Aug. 2010	USDA	10C	Zapata	0	0
Aug. 2010	USDA	11C	Zapata	+	+
Aug. 2010	USDA	12C	Zapata	0	0
Aug. 2010	USDA	13C	Zapata	0	0
Aug. 2010	USDA	14C	Zapata	0	0
Aug. 2010	USDA	15C	Zapata	0	0
Aug. 2010	USDA	1I	Zapata	0	0
Aug. 2010	USDA	2I	Zapata	0	0
Aug. 2010	USDA	3I	Zapata	0	0
Aug. 2010	USDA	4I	Zapata	0	0
Aug. 2010	USDA	W3	Zapata	0	0
Aug. 2010	USDA	Y15	Zapata	0	0
Aug. 2010	USDA	R2	Zapata	0	0
Aug. 2010	USDA	R8	Zapata	0	0
Aug. 2010	USDA	R11	Zapata	0	0
Aug. 2010	USDA	R15	Zapata	0	0

Table 3 continued

Aug. 2010	USDA	R23/25	Zapata	0	0
Aug. 2010	USDA	R31	Zapata	0	0
Aug. 2010	USDA	R32	Zapata	0	0
Aug. 2010	USDA	R33	Zapata	0	0
Aug. 2010	USDA	R35	Zapata	0	0
May 2010	TX AgriLife	W2	Webb	0	0
May 2010	TX AgriLife	W3	Webb	0	+
May 2010	TX AgriLife	W4	Webb	0	0
May 2010	TX AgriLife	W5	Webb	0	0
May 2010	TX AgriLife	W6	Webb	0	0
May 2010	TX AgriLife	W7	Webb	+	0
May 2010	TX AgriLife	W8	Webb	0	0
May 2010	TX AgriLife	W9	Webb	0	0
May 2010	TX AgriLife	W11	Webb	0	0
May 2010	TX AgriLife	W12	Webb	0	0
May 2010	TX AgriLife	W13	Webb	0	0
May 2010	TX AgriLife	W14	Webb	0	0
May 2010	TX AgriLife	W15	Webb	0	0
May 2010	TX AgriLife	W16	Webb	0	0
May 2010	TX AgriLife	W17	Webb	0	0
May 2010	TX AgriLife	W18	Webb	0	0
May 2010	TX AgriLife	W19	Webb	0	0
May 2010	TX AgriLife	W20	Webb	0	0
May 2010	TX AgriLife	W21	Webb	0	+
May 2010	TX AgriLife	W22	Webb	+	0
May 2010	TX AgriLife	W23	Webb	+	+
May 2010	TX AgriLife	W24	Webb	0	0
May 2010	TX AgriLife	W25	Webb	+	0
May 2010	TX AgriLife	W26	Webb	+	0
May 2010	TX AgriLife	L1	Webb	0	0
May 2010	TX AgriLife	L2	Webb	0	0
May 2010	TX AgriLife	L3	Webb	0	0
May 2010	TX AgriLife	L4	Webb	0	0
May 2010	TX AgriLife	L5	Webb	0	0
May 2010	TX AgriLife	L6	Webb	0	0
May 2010	TX AgriLife	L7	Webb	0	0
May 2010	TXAgriLife	L8	Webb	0	0
May 2010	TX AgriLife	L9	Webb	+	0
May 2010	TX AgriLife	L10	Webb	0	0
May 2010	TX AgriLife	L11	Webb	+	0
May 2010	TX AgriLife	L12	Webb	0	0
May 2010	TX AgriLife	L13	Webb	0	0
May 2010	TX AgriLife	L14	Webb	0	0

Table 3 continued					
May 2010	TX AgriLife	L15	Webb	0	0
May 2010	TX AgriLife	L16	Webb	0	0
May 2010	TX AgriLife	L17	Webb	0	0
May 2010	TX AgriLife	L18	Webb	0	0
May 2010	TX AgriLife	L19	Webb	0	0
May 2010	TX AgriLife	20	Webb	0	0
May 2010	TX AgriLife	L21	Webb	0	0
May 2010	TX AgriLife	L22	Webb	0	0
May 2010	TX AgriLife	L23	Webb	0	0
May 2010	TX AgriLife	L25	Webb	0	0
May 2010	TX AgriLife	L26	Webb	0	0
May 2010	TX AgriLife	L27	Webb	0	0
May 2010	TX AgriLife	L28	Webb	+	0
May 2010	TX AgriLife	L29	Webb	0	0
May 2010	TX griLife	L30	Webb	0	0
May 2010	TX AgriLife	L31	Webb	0	0
May 2010	TX AgriLife	L32	Webb	0	0
May 2010	TX AgriLife	L33	Webb	0	0
May 2010	TX AgriLife	L34	Webb	0	0
May 2010	TX AgriLife	L35	Webb	0	0
May 2010	TX AgriLife	L36	Webb	0	0
May 2010	TX AgriLife	L37	Webb	0	0
May 2010	TX AgriLife	L38	Webb	0	0
May 2010	TX AgriLife	L39	Webb	+	0
May 2010	TX AgriLife	L40	Webb	0	0

highly supported relations between clones of the same WTD parasite DNA samples such as 4C clones 1 and 3 which were provided by the USDA from Zapata County, as well as WTD parasite sample SL2 clones 2 and 3, provided by APHIS from Zapata County. A highly supported relationship was found between WTD parasite DNA sample 2C, clones 1 and 2, from Zapata County and BboMer1, which was a bovine *B. bovis* rDNA isolate sequence used as a reference. T2BoChr3 and T2BoChr4, which each represent one copy of the bovine T2bo *B. bovis* rDNA isolate reference sample (*Babesia bovis* genome

project http://www.vetmed.wsu.edu/researchvmp/program-in-genomics), were placed on a branch separate from WTD parasite sequences, however, this was not heavily supported by the bootstrap tree indicating they showed very close genetic similarity to the WTD *B. bovis* parasite sequences (Fig.4). The phylogram, however, better illustrates the relationships since the length of connecting lines corresponds to the genetic distances among the sequences. The two copies found in the T2Bo *B. bovis* type sequence (http://www.vetmed.wsu.edu/researchvmp/program-in-genomics, *Babesia bovis* genome project), T2BoChr3 and T2BoChr4, are very similar to and very closely related to the WTD *B. bovis* parasite sequences.

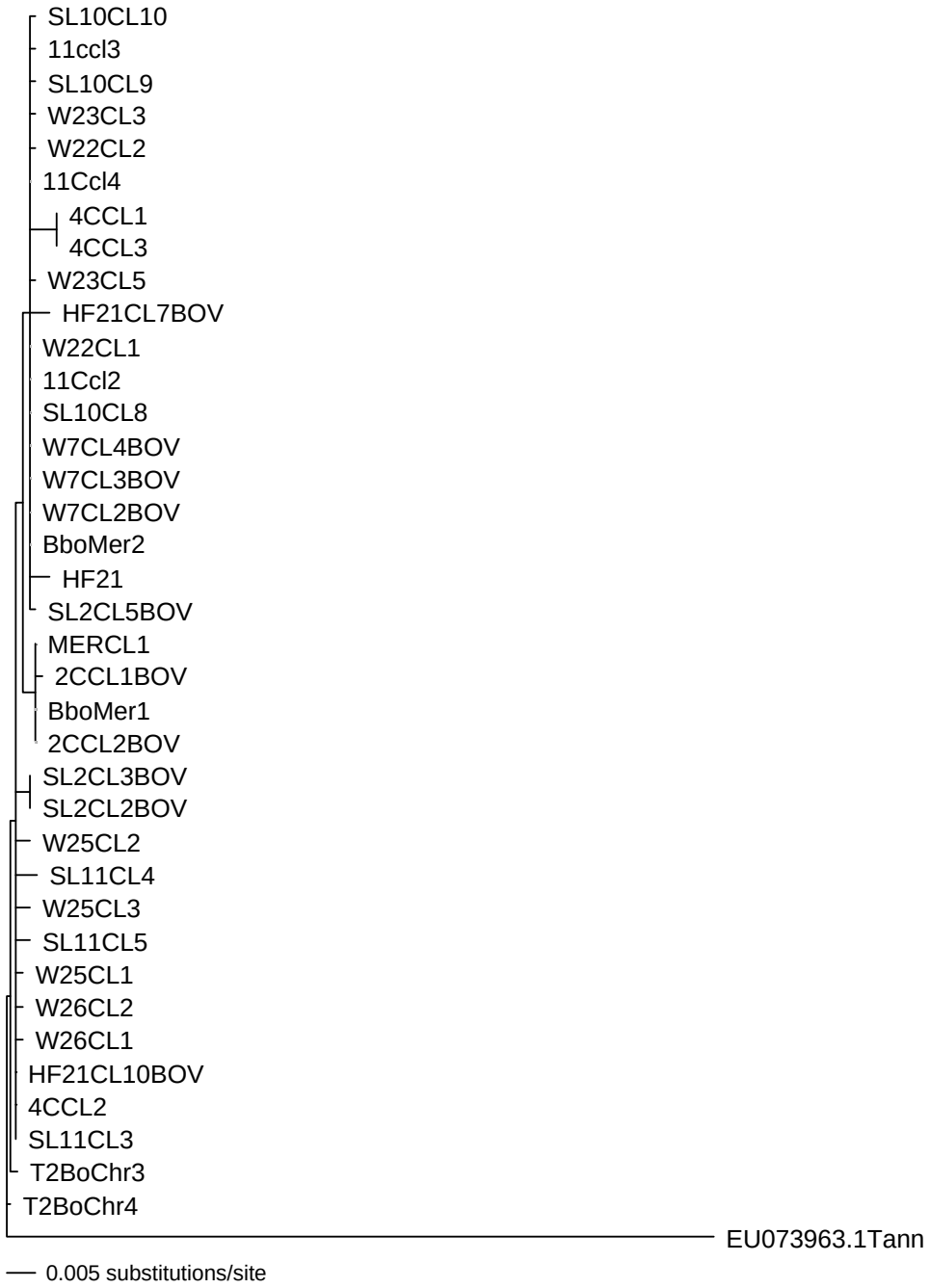

Fig 3. Neighbor-joining phylogenetic tree showing bovine *Babesia bovis* and cervine *B. bovis*-like 18S rDNA cloned sequences. White-tailed deer parasite sequences clustered alongside cattle *Babesia bovis* sequences and short genetic distances within the formed clades were observed implicating strong genetic commonalities among the parasite sequences from both hosts.

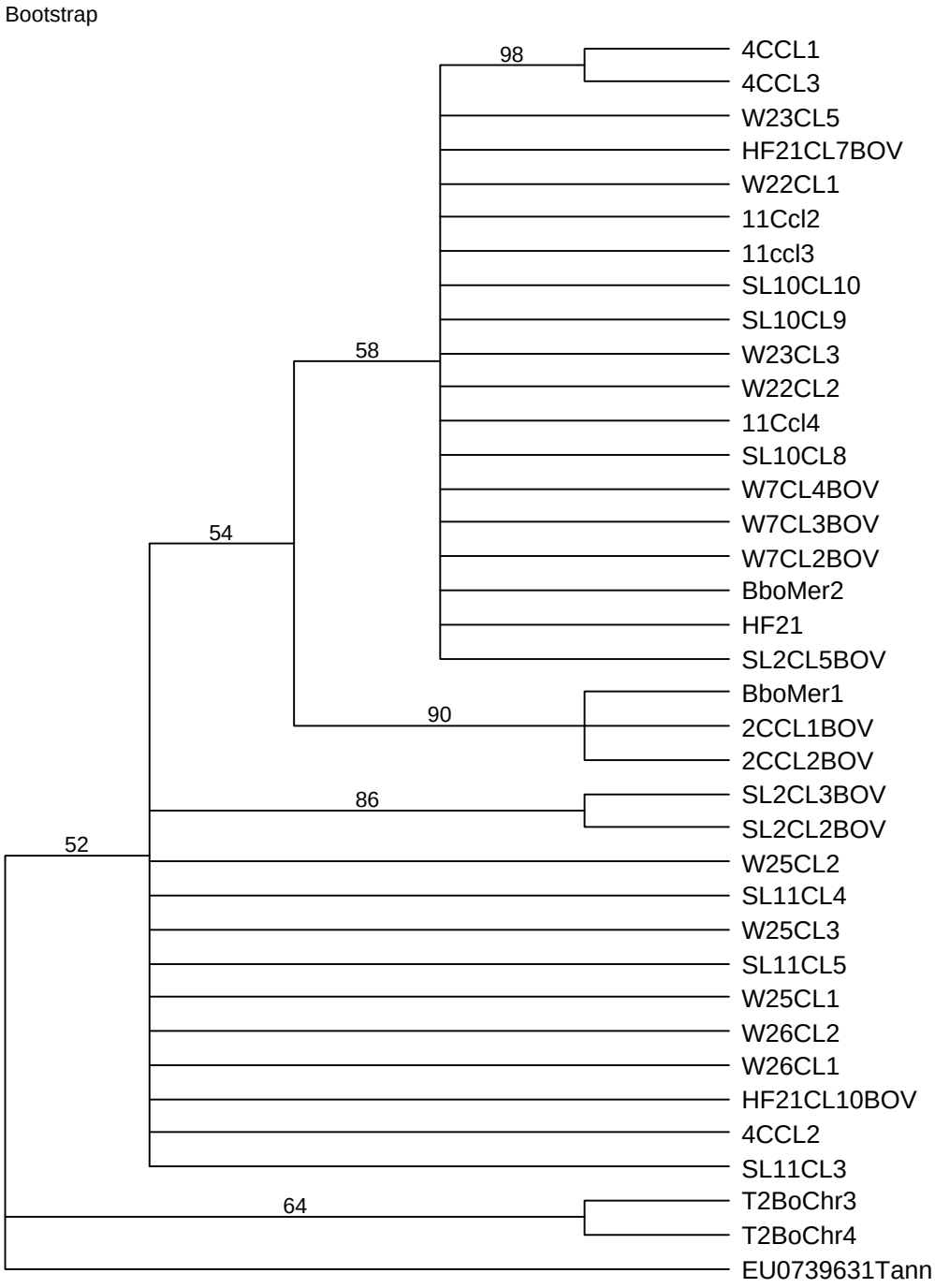

Fig 4. Neighbor-joining bootstrap tree showing bovine *Babesia bovis* and cervine *B. bovis*-like 18S rDNA cloned sequences. Parasite sequences from white-tailed deer blood samples clustered with known *Babesia bovis* sequences from cattle showing close identity among sequences.

Sequences from *B. bigemina* positive samples showed 99% identity to *B. bigemina* 18S rDNA sequences of cattle origin in the GenBank database (EF458201.1, EF458203.1, EF458204.1, and EU109716.1). When the WTD parasite 18S rDNA sequences were compared to the bovine *B. bigemina* reference sequences in the constructed phylogenetic and bootstrap trees, very short genetic distances were found between the two groups (Figs. 5 and 6). These short genetic distances indicate that the WTD *B. bigemina* sequences from this study are closely related to the bovine *B. bigemina* isolates used as reference sequences. In the bootstrap tree (Fig. 5) WTD parasite sequences cluster with the two reference samples, EF458201B.big and EF458204B.big, but are found on a separate branch. The bootstrap model supports the separation only 64%, indicating that these two branches are related and this is the closest relationship seen in this tree. The phylogram (Fig. 6) shows a close relationship between WTD *B. bigemina* sequences W23 clones 3 and 4, which were from Webb County. There is also notable identity among WTD *B. bigemina* sequences W23 clone 2, W3 clones 1, 5 and 7, and 11C clone 2. The W23 and W3 samples were from Webb County while the 11C sample was from Zapata County. The previously identified *B. bigemina*-like sequences from WTD H8 (Holman et al., 2011) sequences show some genetic distance, branching separately from the others.

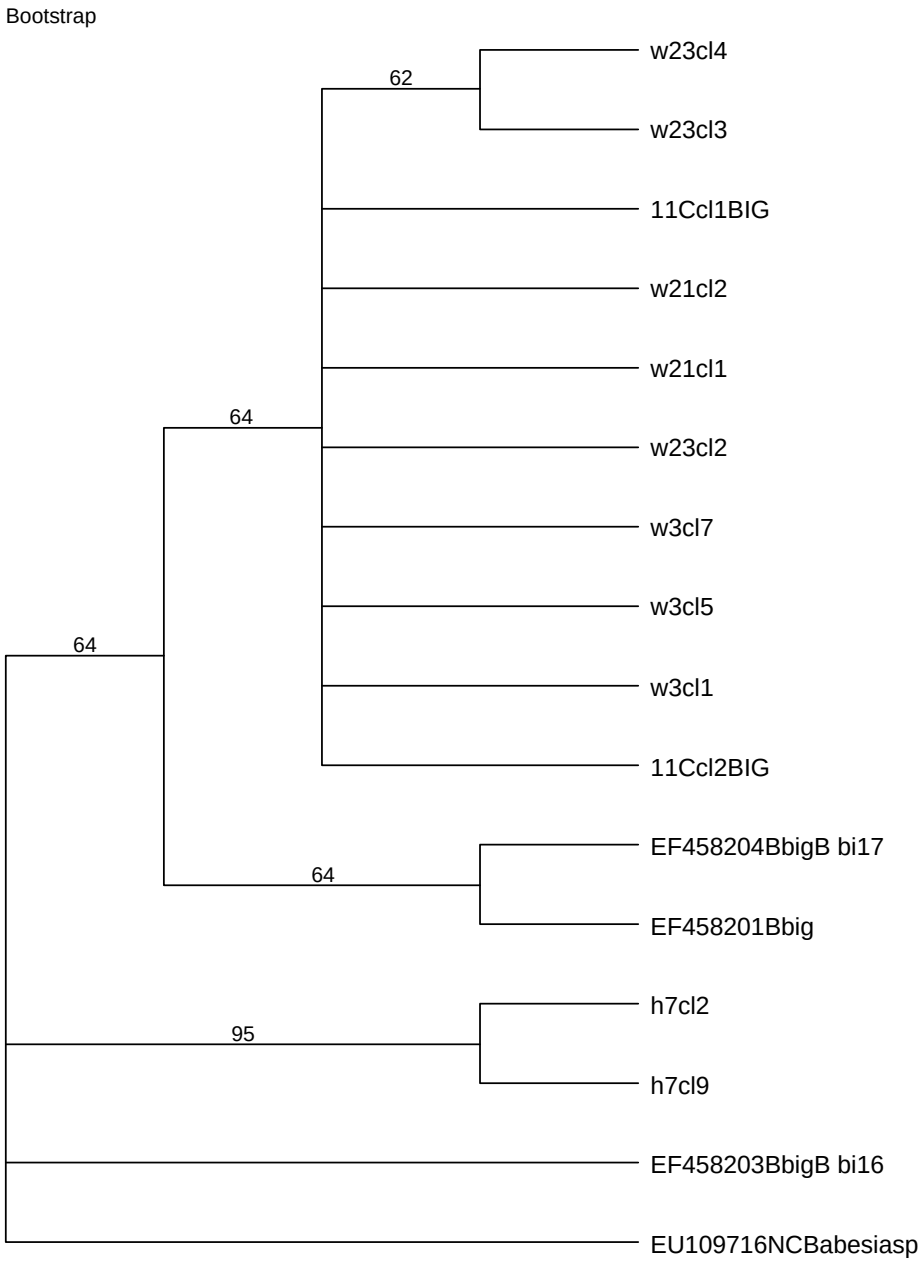

Fig 5. Neighbor-joining bootstrap tree showing bovine *Babesia bigemina* and cervine *B. bigemina*-like 18S rDNA cloned sequences. Parasite sequences isolated from white-tailed deer blood samples clustered with parasite sequences isolated from cattle blood samples showing close identity among sequences.

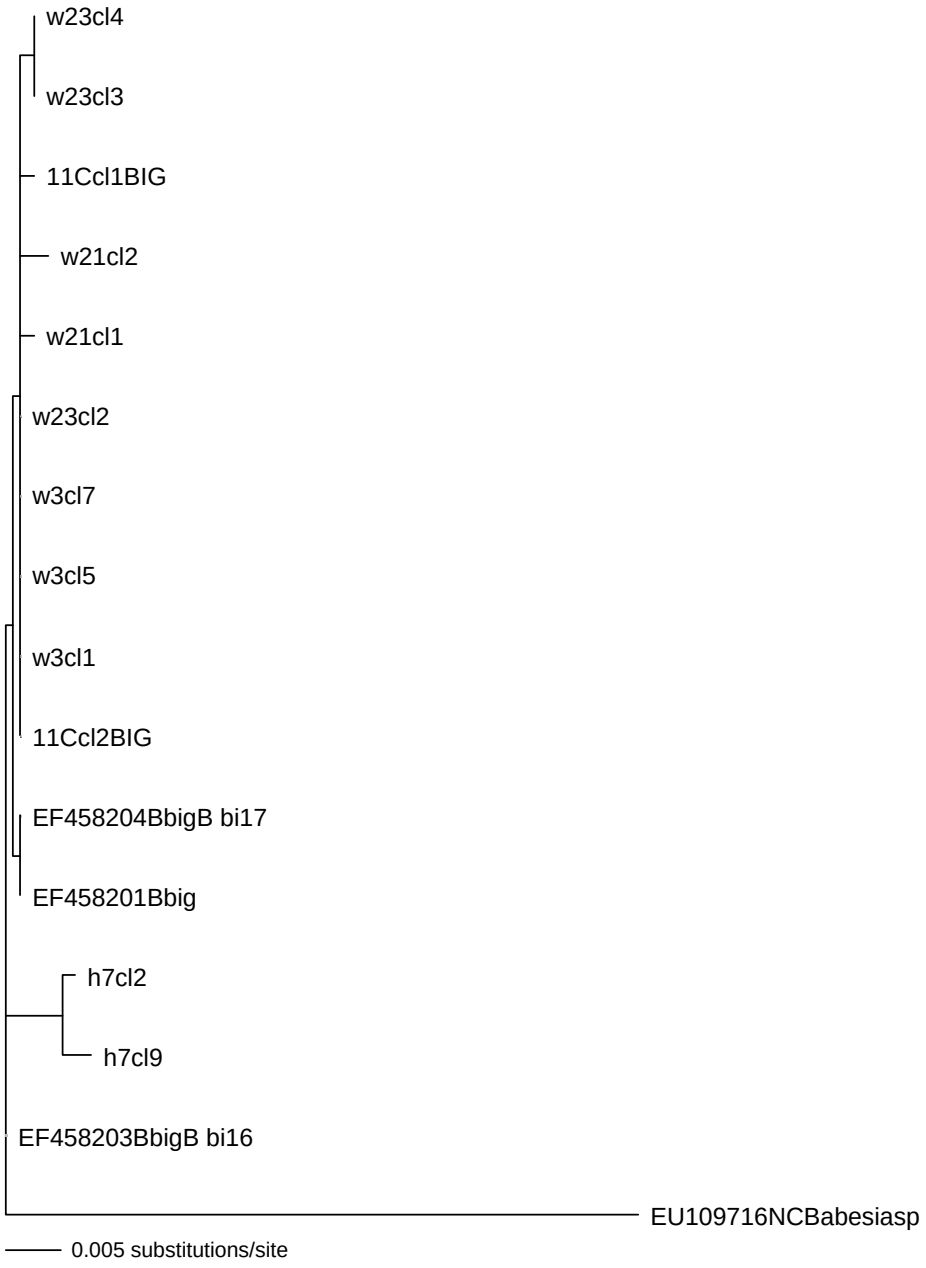

Fig 6. Neighbor-joining phylogenetic tree showing bovine *Babesia bigemina* and cervine *B. bigemina*-like 18S rDNA cloned sequences. White-tailed deer parasite sequences clustered alongside cattle parasite sequences and short genetic distances within the formed clades were observed implicating strong genetic commonalities among sequences isolated from both hosts.

The ITS sequences from *B. bovis* PCR positive WTD samples also showed 99% identity to ITS sequences from *B. bovis* of cattle origin in the GenBank database. WTD parasite sequences were clustered alongside bovine *B. bovis* ITS sequences on highly supported branches in the bootstrap tree (Fig. 7) rather than forming a clade of only WTD parasite sequences. This indicates the sequences are essentially indistinguishable to those of known bovine ITS *B. bovis* isolates. The Webb County WTD parasite clones of samples L39 and L28 were clustered on the same branch while WTD parasite clones of samples L9 and L11 were clustered on a separate branch. L9 clone 4 and L11 clone 6 showed a strong relationship and were clustered together on a small branch rooted from the branch containing all other L9 and L11 clones. The genetic distances shown in the phylogram (Fig. 8) indicate the two groups, L9/ L11 and L28/L39, have minor differences among themselves but strong identity to the bovine ITS *B. bovis* isolate sequences.

Sequencing did not prove to be 100% reliable. Samples were initially run in one direction, using either M13 Forward primers or M13 Reverse primers. Sequencing mistakes would occur causing all or portions of the sequences to be unreadable so the samples were sent in once more to be run in the opposite direction using the opposite primer. Occasionally, however, a completely unreadable set of data was produced from sequencing and these samples were not able to be used for research purposes. Three samples were unable to be used in this research due to these circumstances; they include sample 5C clone 1 from Zapata County and samples L39 clone 1 and L9 clone 2 from Webb County.

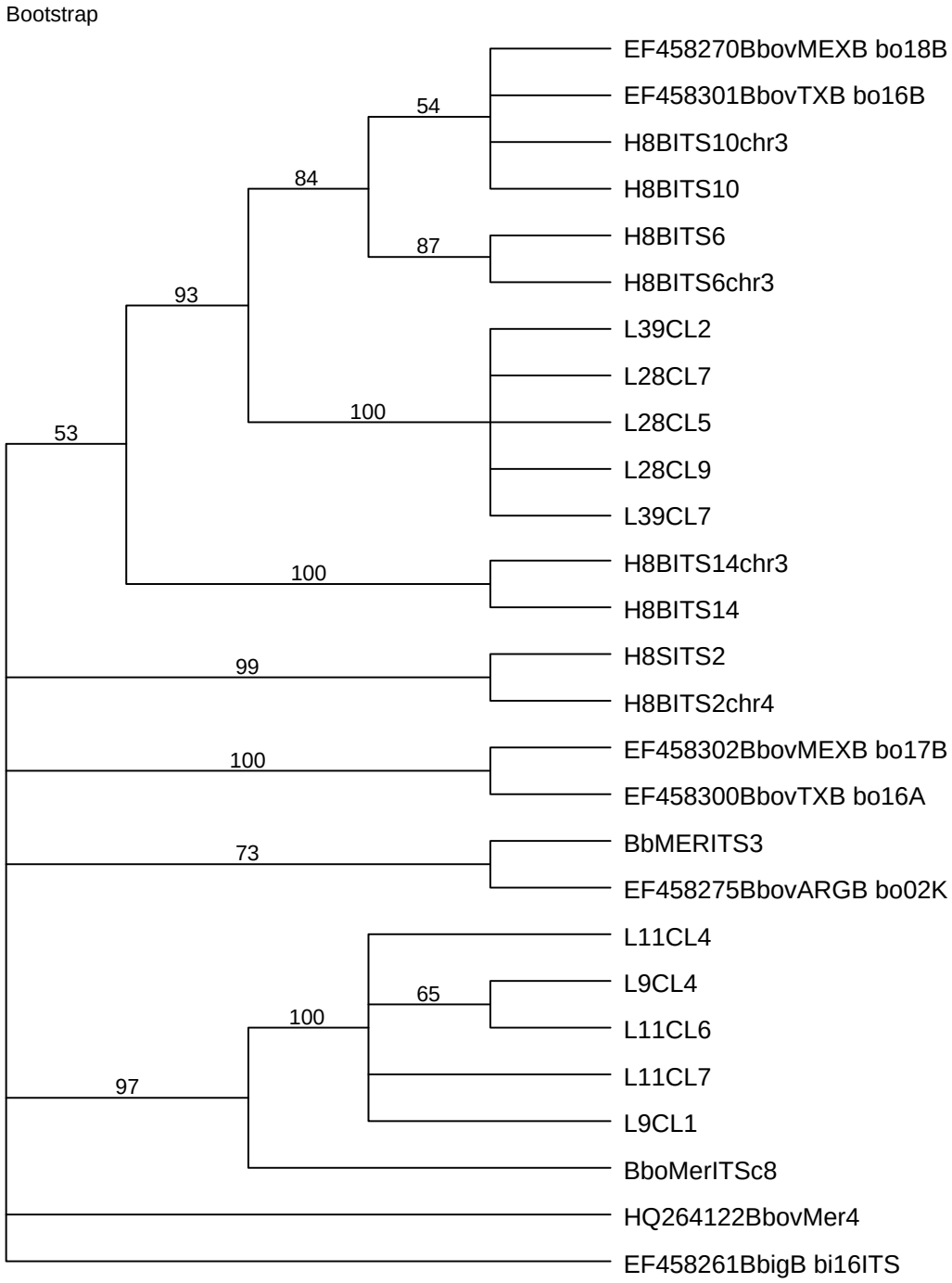

Fig 7. Neighbor-joining bootstrap tree showing bovine *Babesia bovis* ITS and cervine *B. bovis*-like ITS cloned sequences. Parasite sequences from white-tailed deer blood samples clustered with *B. bovis* sequences from cattle blood samples showing close identity among sequences.

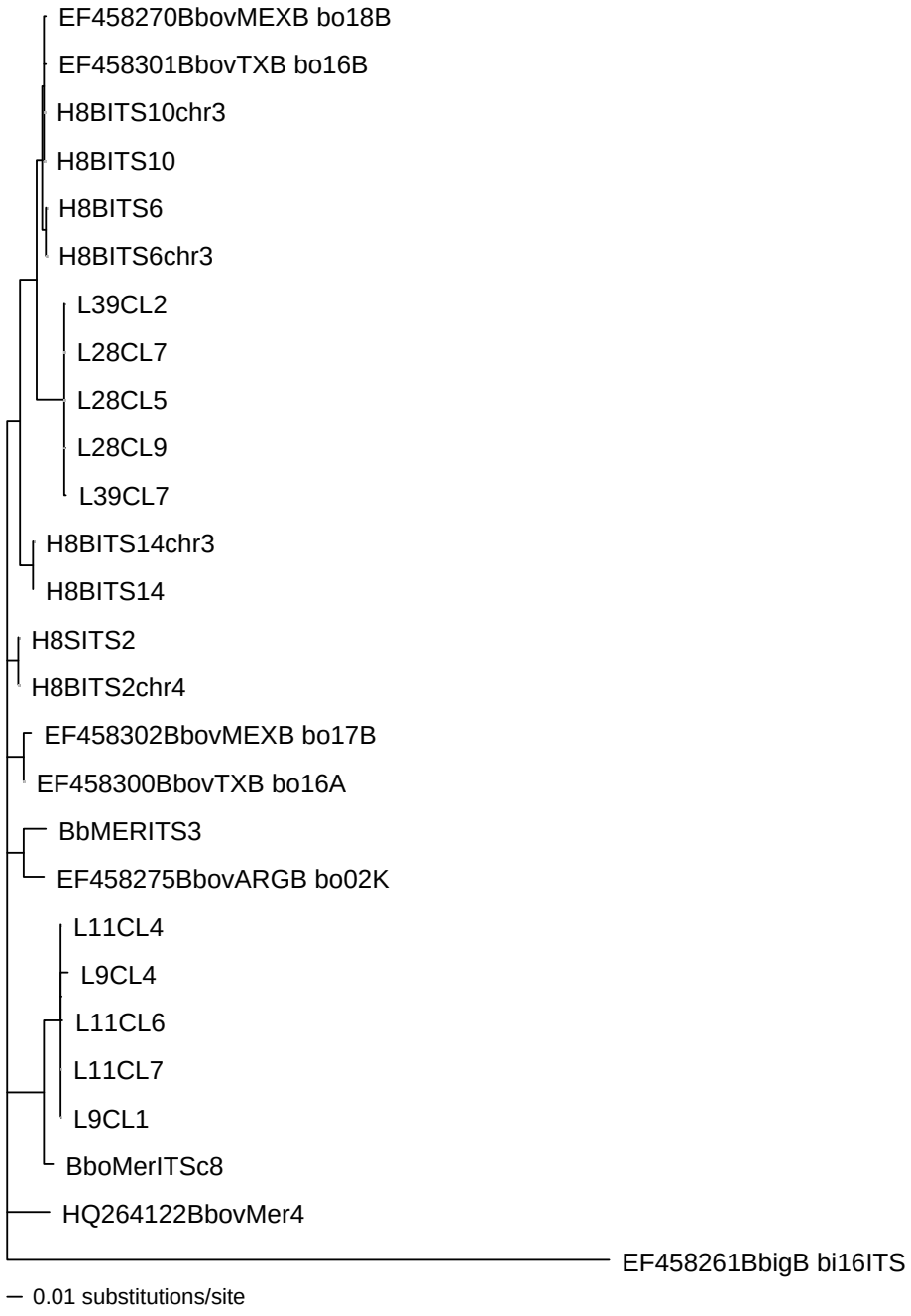

Fig 8. Neighbor-joining phylogenetic tree showing bovine *Babesia bovis* ITS and cervine *B. bovis*-like ITS cloned sequences. White-tailed deer parasite sequences clustered alongside cattle *B. bovis* sequences and short genetic distances within the formed clades were observed implicating strong genetic commonalities among parasite sequences from both hosts.

CHAPTER IV

CONCLUSIONS

The aim of this study was to determine whether or not white-tailed deer serve as alternate hosts to *Babesia bovis* and *Babesia bigemina* parasites which are known to infect cattle, causing bovine babesiosis. From rDNA sequence analyses (Figs. 3-8), it becomes obvious white-tailed deer carry *Babesia* spp. that are phylogenetically very closely related to *B. bovis* and *B. bigemina* parasites of cattle origin. Sequences from deer parasites clustered alongside those of cattle parasites in bootstrap and phylogenetic trees constructed, and short genetic distances were observed in all three phylogenetic trees indicating strong genetic commonalities among the samples.

Previous studies of *Babesia* spp. confirmed the presence of *B. bovis* and/or *B. bigemina* in white-tailed deer in Mexico; however, these studies did not ascertain the phylogenetic relationships among white-tailed deer parasites and parasites derived from cattle isolates (Cantu et al., 2007, 2009). Although genetic identity among *Babesia* parasites from the two hosts, white-tailed deer and cattle, was established in the current study, the results from this study cannot definitively prove the *Babesia* spp. infecting white-tailed deer are able to be transmitted through tick vectors from the deer hosts to cattle. Infection studies that show cattle are susceptible to the parasites carried by white-tailed deer are needed to definitively identify these parasites as identical to bovine *B. bovis* and *B. bigemina.* This could be done by demonstrating transmission of *B. bovis*- and/or *B. bigemina*-like

parasites from white-tailed deer hosts into *Rhipicephalus (Boophilus)* spp. tick vectors and, ultimately, transmission of these white-tailed deer *Babesia* spp. from the infected ticks to susceptible cattle hosts.

The possibility and magnitude of economic strains on the cattle industry alone are reason enough to continue studies of white-tailed deer as reservoirs of infection for the parasites which cause bovine babesiosis. Bovine babesiosis will remain a threat to both the United States and Mexico if the weight of *B. bovis* and *B. bigemina* in white-tailed deer is underestimated and ignored.

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